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Canberra

I, JONNE YABSLEY, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PQ 6973 for a patent by GRADIPORE LIMITED filed on 18 April 2000.

WITNESS my hand this
Twenty-third day of April 2001

J R Yabsley

JONNE YABSLEY
TEAM LEADER EXAMINATION
SUPPORT AND SALES

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AUSTRALIA

Patents Act 1990

Gradipore Limited

PROVISIONAL SPECIFICATION

Invention Title:

Separation apparatus

The invention is described in the following statement:

Field of the Invention

This invention relates to separation apparatus and in particular, to apparatus for purification of macromolecules in solution.

Background of the Invention

European patent No 352286 concerns the separation of macromolecular solutes by a process known as electrophoretic separation or electrophoresis, in particular, fixed boundary electrophoretic separation. In fixed boundary electrophoresis as described in EP 352286, a semi-permeable membrane (hereinafter referred to as a separation membrane), acts to separate two streams of liquid carrying macromolecular solutes such as proteins, known as the upstream and the downstream. The streams flow between charged electrodes and at least one macromolecular solute migrates across the membrane from one stream to the other stream under the influence of the electric field. The apparatus of EP 352286 also includes flow paths for buffer solutions and further semi-permeable membranes, hereinafter referred to as restriction membranes, disposed either side of the separation membrane between the electrodes and the separation membrane to separate the buffer flow paths from the upstream and downstream. The restriction membranes allow the passage of ions but not of the relatively larger macromolecules.

International patent application PCT/AU99/00732 develops and improves the ideas and principals disclosed in EP 352286 and provides refinements and improvements to the apparatus which make the technology easier to use and operate.

In particular, it provides a system in which the separation and restriction membranes and upstream and downstream are contained in a removable and replaceable cartridge. Although that specification provides a substantial improvement over EP 352286, electrophoresis cannot satisfactorily be used for separating very small samples. Even an apparatus of reduced size such as is described in PCT/AU99/00732 is unable to separate very small samples, with the smallest practicable sample size being around 6 mL. Therefore, for smaller samples, other separation methods such as chromatography or gel electrophoresis, have to be used. However, such other methods are time consuming. For example, in gel electrophoresis, the separation in the gel is very slow, taking several hours and further time is wasted in subsequently extracting (eluting) the separated molecules from the gel. A further problem arises in that molecules tend to elongate and denature

when separated in a gel in comparison with a free flow electrophoresis separation.

Prior art electrophoretic separation apparatus and methods have been developed for processing large sample volumes and are not suitable to treat small volumes. Furthermore, the ratio of sample to electrophoresis membrane surface area is usually greater than 2.5 mL/cm^2, typically around 5 mL/cm^2, which results in large dead volumes and the need to re-circulate buffers and samples to reduce heating and prevent clogging of membranes.

The present invention seeks to alleviate the problems of the prior art and in particular, to provide a separation method and apparatus suitable for use with relatively small sample volumes.

Summary of the Invention

In a first aspect, the present invention provides a method of separating macromolecules in small volumes of solution across an electrophoresis separation membrane under the influence of an electric field, wherein macromolecules transfer across the separation membrane from a volume of liquid in an upstream chamber on one side of the separation membrane to a volume of liquid in a downstream chamber on an opposite side of the membrane, characterised in that there is substantially no recirculation of the volumes of liquid.

Preferably, the small volume is less than about 5 mL, preferably about 2 mL or less. The invention is particularly suitable for separating samples of around 0.1 to 0.5 mL.

The present inventors have found that a ratio of sample to separation or restriction membrane surface area of less that about 1 mL/cm^2 is required for the present invention. More preferably, the ratio is 0.5 mL/cm^2, and even more preferably the ratio is 0.1 mL/cm^2.

In a second aspect, the present invention provides a apparatus for separating macromolecules in small volumes, the apparatus comprising:-

(a) outer buffer chambers;

(b) electrodes housed in the outer buffer chambers; and

(c) a cartridge positioned between the outer buffer chambers, the cartridge defining at least one upstream chamber and at least one downstream chamber separated by an electrophoresis separation membrane, the at least one upstream and downstream chambers being separated from the outer buffer chambers by a restriction membrane positioned either side of the

electrophoresis membrane allowing flow of ions into and out of the chambers under the influence of an electric field, wherein in use there is substantially no recirculation of liquid in the chambers or buffer.

Preferably, the small volume is less than about 5 mL, preferably about 2 mL or less. The invention is particularly suitable for separating samples of around 0.1 to 0.5 mL.

The present inventors have found that a ratio of sample to separation membrane surface area of less that about 1 mL/cm^2 is required for the present invention. More preferably, the ratio is 0.5 mL/cm^2, and even more preferably the ratio is 0.1 mL/cm^2.

In one preferred form, the cartridge is adapted to be removable from a buffer tank. When placed the cartridge is placed in the tank, the outer buffer chambers are formed. The removable cartridge may include handles for ease of removal or placement.

The cartridge may define several upstream and downstream chambers by comprising several electrophoresis separation membranes between each chamber.

Upper parts of the chambers may be wider than lower parts of the chambers for ease of loading the sample.

In a third aspect, the present invention provides a method for desalting or dialysing a small volume sample containing at least one macromolecule, the method comprising the steps of:

(a) placing a sample in a sample chamber adapted to receive a small volumes and positioned between outer buffer chambers, the sample chamber being formed by an upper and lower restriction membrane; and

(b) applying an electric field to the sample in the sample chamber such that salts in the sample move to the buffer in the outer buffer chambers while the at least one macromolecule is substantially retained in the sample chamber, wherein there is substantially no re-circulation of liquid in the sample chamber or buffer in the outer buffer chambers.

Preferably, the small volume is less than about 5 mL, preferably about 2 mL or less. The invention is particularly suitable for separating samples of around 0.1 to 0.5 mL.

The present inventors have found that a ratio of sample to restriction membrane surface area of less that about 1 mL/cm^2 is required for the present

invention. More preferably, the ratio is 0.5 mL/cm^2, and even more preferably the ratio is 0.1 mL/cm^2.

The upper and lower restriction membranes allow the movement of ions and small molecular weight compounds but do not allow the movement of the one or more macromolecules to be desalted.

In a fourth aspect, the present invention provides an apparatus for desalting or dialysing small volumes of macromolecules, the apparatus comprising:

(a) outer buffer chambers;

(b) electrodes housed in the buffer chambers; and

(c) at least one sample chamber positioned between the outer buffer chambers and adapted to receive a small volume, the sample chamber formed by a pair of restriction membranes which allow the flow of ions and small molecular weight compounds to buffer on either side of the sample chamber under the influence of an electric field, wherein in use there is substantially no re-circulation of liquid in the sample chamber or buffer in the outer buffer chambers.

Preferably, the small volume is less than about 5 mL, preferably about 2 mL or less. The invention is particularly suitable for separating samples of around 0.1 to 0.5 mL.

The present inventors have found that a ratio of sample to restriction membrane surface area of less that about 1 mL/cm^2 is required for the present invention. More preferably, the ratio is 0.5 mL/cm^2, and even more preferably the ratio is 0.1 mL/cm^2.

In a particularly preferred embodiment of the second and fourth aspects of the present invention, the chambers are formed with an open top in use with the separation and restriction membranes oriented substantially vertically and the electric field passing generally horizontally from one electrode to another so that the sample and buffers can be simply and easily loaded into the respective chambers from above. This arrangement is particularly advantageous in terms of the ease of use of the apparatus.

The chambers may be provided as a removable cartridge which is adapted to locate in a buffer tank which houses the electrodes and electrophoresis buffers.

Surprisingly, it has been found that it is possible to conduct electrophoresis and other similar separation methods such as dialysis,

desalting utilising static small sample volumes, without re-circulation or flow of the samples which was previously thought necessary in order to achieve a good separation. Other methods and apparatus require re-circulation or mixing to cool the sample to prevent overheating and consequent denaturing or destruction of macro molecules in the sample. Instead, it has been found by the present inventors that with small sample volumes and apparatus adapted to take small volumes, a "static" separation without re-circulation, can be accomplished satisfactorily and beneficially, in a surprisingly short period of time.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

In order that the present invention may be more clearly understood preferred forms will be described with reference to the following drawings.

Brief Description of the Drawings

The invention will now be described by way of example only and with reference to the accompanying drawings in which:

Figure 1a is a schematic drawing illustrating the basic principal of the present invention;

Figure 1b is a schematic drawing illustrating a variant of the basic principal shown in Figure 1a;

Figure 2 is an exploded view of a first embodiment of an apparatus embodying the present invention;

Figure 3 is an exploded view of a second embodiment of the present invention;

Figure 4 is an assembled view of the embodiment of Figure 3;

Figure 5 is a schematic drawing illustrating the use of the present invention for dialysis, also known as desalting;

Figure 6 is a schematic drawing illustrating multiple dialysis mode;

Figure 7 is a schematic drawing illustrating a variant of the arrangement illustrated in Figure 1b in which multiple different separation membranes are provided;

Figure 8 is a schematic drawing illustrating the use of multiple parallel separation membranes;

Figure 9 is a schematic drawing illustrating the use of multiple upstream and downstream chambers and multiple outer buffer chambers;

Figure 10 is a schematic drawing illustrating a full sized array using multiple separation membranes having multiple upstream chambers and five parallel rows of multiple downstream chambers;

Figure 11 illustrates charge-based separation;

Figure 12 illustrates charge and size based separation;

Figure 13 illustrates concentration; and

Figure 14 illustrates dialysis.

Detailed Description of Preferred Embodiments

Before describing the preferred embodiments in detail, the principal of operation of the apparatus will first be described.

An electric field applied to charged molecules including macromolecules such as proteins in solution, will cause the molecules to move to one of the electrodes. If the protein has a positive charge, it will move to the negative electrode (cathode). Conversely, a negatively-charged protein will move to the positive electrode (anode).

In the apparatus of the present invention for separating macromolecules, an electrophoretic separation membrane is placed in the electric field and molecules are selectively transported between the upstream and the downstream chambers. The particular separation membrane used will vary for different applications and generally has a relatively large, but well defined, pore size. The upstream and downstream chambers are isolated from the electrodes by buffer streams and by two restriction membranes. The restriction membranes allow the movement of relatively small molecules and ions up to a molecular mass of about 3,000 Dalton.

There are four modes of operating the apparatus of the present invention. These are set out below and are illustrated in Figures 11 to 14, respectively. It should be noted that the Figures show free-flow electrophoresis and suggest circulation/flow of the upstream and downstream, which in the present invention does not take place.

1. Charge-based separation

In principal, any two molecules with different pIs can be separated by carrying the separation out at a pH between the two pIs. The pI is the pH of a solution in which the molecule has neutral charge, thus by changing the pH

of the solution in which a molecule is present, the effective charge of that molecule can be changed.

Thus in a solution with a pH between the two pIs, one molecule will have a positive charge, and will move towards the cathode and be contained in the upstream. The other molecule will have a negative charge and will be contained in the downstream as it moves towards the anode. Figure 11 illustrates charge-based separation.

2. Sized Based Separation

Components with different molecular masses can be separated on the basis of pore size of the electrophoresis separation membrane. Figure 12 shows an example of separation based on size. Two proteins which are both negatively charged are separated because the larger molecules are unable to migrate through the smaller pores of the separation membrane. Careful combination of pore size and pH can often allow the isolation of a single component from a complex mixture in one electrophoresis run.

Examples are monoclonal antibodies (Mab) from ascitic fluid and ovalbumin, lysozyme, or avidin from egg white, and fibrinogen from plasma.

3. Concentration

Figure 13 illustrates concentration, which utilises a large pore size separation membrane (1,000 kDa). The large pore sizes enable the rapid transportation of proteins across the separation membrane from a large volume upstream solution to a small volume downstream solution. In this process a pH is selected in which all of the desired proteins will have the same charge. Typically, pH 8.3 is selected since most proteins will have a negative charge at this pH. In many applications, purification can be achieved at the same time as concentration.

4. Dialysis

Figure 14 illustrates dialysis also known as desalting. The apparatus of the present invention can also be used for desalting. The separation membrane is not necessary for dialysis, which can be performed with or without this membrane. The ions are removed from the sample by passing through the restriction membranes and then are washed away by the outer buffer chambers. This process also occurs during standard fractionation or concentration, but in these cases the ions are re-circulated. When being used for extensive dialysis, the buffer where the ions collect, should be exchanged for fresh buffer solution at regular intervals.

EXAMPLES

Now turning to the specific embodiments of the invention and referring to the drawings, Figure 1 is a schematic view illustrating an electrolytic cell 10. The cell consists of four chambers 12, 14, 16 and 18, specifically outer chambers 12 and 18 and inner chambers 14 and 16. A negative electrode 20 is located in outer chamber 12 and a positive electrode 22 is located in outer chamber 18. The inner chambers 14, 16 are the "upstream" and the "downstream" chambers respectively. A restriction membrane 24 separates the downstream chamber 16 from the outer chamber 18 containing the positive electrode 22. A similar restriction membrane 26 separates the upstream chamber 14 from the outer buffer chamber 12 which contains the negative electrode 20. A separation membrane 28, separates the upstream chamber 14 from the downstream chamber 16. In use, the outer chambers contain buffer and the restriction membrane defines holes or pores which are large enough to allow the flow of ions, but not large enough to allow the flow of macromolecules.

Although chambers 14 and 16 are referred to as the "upstream" and the "downstream" chambers, there is no flow or recirculation of the contents of the upstream or downstream chambers. The samples in the upstream and downstream chambers remain essentially static, although clearly there will be some random movement of individual molecules, and also transfer of molecules across the restriction and separation membrane due to the electric field generated by the electrodes due to the mechanisms described above.

Figure 1b shows a variant of the arrangement shown in Figure 1a in which instead of a single chamber 14 and 16 either side of the separation membrane, the upstream chamber is divided into a series of chambers 14a, 14b .... 14j and the downstream is divided into a series of ten chambers 16a, 16b .... 16j. This arrangement allows separations of ten small samples to be carried out at the same time. It is to be noted that each separation shares the same buffer.

Figure 2 shows an exploded view of a first physical embodiment of an apparatus according to the present invention. The apparatus comprises two body portions 50 and 52. There are three generally U-shaped channels 54, 56, 58 which extend from a rear wall 60 of the first body portion 50 to the front of the body portion. The three channels 54, 56, 58 are contiguous and parallel, and channels 54 and 56 share a side wall as do channels 56 and 58.

The end walls of the group of three channels terminate in a plane which projects from the front wall of the first body portion 50.

The second body portion 52 also provides three channels 62, 64, 66 which extend from its rear wall 68. Those channels are also contiguous and channels 62 and 64 share a side wall as do channels 64 and 66. The cross-section of the three channels 62, 64 , 66 matches that of channel 54, 56, 58. The end walls of the group of three channels define a recess 72 which is of a size and shape to receive the end walls of the projecting channels from body portion 50 with a small amount of clearance. When the two body portions 50,52 are pushed together the end walls of the channels do not meet but are spaced apart by a relatively small distance of several millimetres. The gap between the end walls forms a space in which the upstream and downstream chambers are located. The channels define the outer chambers which hold the buffer.

Three through holes (73) are provided in each body portion, one either side of the channels and one below the channels which are aligned so that when the holes are aligned and the left and right hand blocks join together, the three channels in the block 52 align with the three channels in the block 50 and their ends locate in the recess 72.

A hole 75 extends transversely across each body portion through the walls of the channels. Both holes receives a platinum wire 74 which is connected to a terminal 76.

An arrangement of chambers similar to that illustrated in Figure 1a but having only three upstream chambers and downstream chambers is provided in the space between the end walls. The chambers are provided by a sandwich construction of a restriction membrane 24, a grid spacer element 78 of the same cross section as the groups of channels, a separation membrane 28, a further grid spacer 78 and a further restriction membrane 26.

An important feature of the apparatus shown, apart from the lack of flow/circulation and the lack of a pump, is that both the buffer chambers and the downstream and upstream chambers are all top loading. That means that the samples and the buffer solution can be simply injected or otherwise dropped into the relevant chambers from above using a syringe or pipette or the like. This makes the apparatus easy to use compared with the traditional electrophoresis apparatus where the separation membrane tends to be

aligned in a horizontal plane in use, rather than a vertical plane and sample loading is more complex.

Figures 3 and 4 illustrate a further embodiment of an apparatus according to the present invention incorporating a removable cartridge 100, which may be disposable. In this embodiment, a separate buffer tank 102 is provided which includes electrodes, not shown, which provide an electric field E passing transversely across the tank as shown by the arrow E. At each longitudinal end of the tank, there is provided a female engagement means 104 of which only one is visible in the Figures, between which the cartridge housing can be engaged in a sliding arrangement.

The cartridge 100 comprises a number of components including, two mating housing components a male component 106 and a female component 108 which are adapted to receive two restriction membranes 26, two grids 110 defining a well structure and a separation membrane 28.

The male cartridge housing component 106 is generally rectangular in cross section and defines a generally U shaped rib 107 which projects outwardly from the sides and base of the housing component. The rib mates in a corresponding recess, not shown, which is defined in the sides and the base of the female housing component. A series of holes 112 extend through the male cartridge housing component from an outer face which is hidden in the Figures to an inner face 114. The holes are generally rectangular. The inner face 114 of the housing component is recessed relative to the projecting rib so as to define a recess for receiving the restriction membrane 26 and the grid 110. The top 116 of the recess is chamfered and slopes away towards the outside of the housing. The upper part of the restriction membrane 26 is also bent to match the angle of chamfer of the recess in the housing component.

The grid defining a well structure is an insert which fits inside the recess in the male housing component. The grid defines a series of ten contiguous well structures or chambers 14a to 14j which in use are the upstream chambers and which are aligned with the ten apertures defined in the cartridge housing components. The upper end of the well structure is open. At the top of the wells, there is a bevelled portion which extends outwardly and matches the chamfer of the top of the recess of the housing component, so that the outer face of the well structure matches the inner face of the cartridge housing and restriction membrane.

The female cartridge housing component is largely a mirror image of the male cartridge housing component apart from the U shaped channel which receives the rib in a snap fit action to lock the male housing to the female housing. The female housing receives an identical restriction membrane and grid to the male housing, the grid defining the downstream chambers 16a.....16j. Also the female housing defines two handles which extend upwardly and outwardly from the cartridge. The cartridge will be typically made in a plastics material and is ideally made sufficiently cheaply that it is disposable, although it would in theory be possible to re-use the cartridge if the cartridge were properly cleaned and the separation membrane removed and replaced after use.

It is to be noted that the upper part of the well structure is wider than the lower part of the well structure so that it acts as a type of funnel which makes it easier to load a sample into the chambers of the well structure. This allows the lower part of the chamber to be narrow so that the sample size can be very small and so that the separation process occurs rapidly due to the small sample size.

The embodiment shown in Figures 3 and 4 has advantages in ease of use over the embodiment shown in Figure 2, although unlike the embodiment shown in Figure 2, it has the restriction that all the downstream and upstream chambers share the same buffer.

The apparatus and method of the present invention may also be applied to dialysis in which case no separation membrane is used, and the upstream and downstream or upstream and downstream chambers form one sample chamber. This arrangement is shown in Figure 5 with Figure 6 illustrating a schematic arrangement showing multiple dialysis with multiple buffers and multiple sample chambers. The positive and negative electrodes may be connected together so that the electric field across the multiple buffer and sample chambers is uniform or alternatively, the electrodes could be insulated from one another and used to provide differing electric fields across the differing chambers.

Figure 7 is a schematic arrangement showing multiple upstream chambers and multiple downstream chambers and multiple separation membranes. This arrangement would be possible with the apparatus shown in Figure 3 provided that the separation membrane were replaced with a

series of ten different separation membranes corresponding to the width of the wells.

Figure 8 is a schematic arrangement showing multiple downstream chambers separated from each other by different separation membranes. In one preferred configuration, the separation membranes have reducing molecular mass cut-offs proceeding further from the upstream chamber. In this configuration, it is possible to separate a complex biomolecule mixture in the upstream chamber into different molecular masses defined by the various separation membranes.

Figure 9 is a schematic arrangement showing an apparatus containing ten separation cells. In this arrangement, it is possible to carry out up to even different separations by adding different buffers to each of the buffer chambers and different samples in the multiple upstream chambers. Upon electrophoresis, the result would be the movement of biomolecules from the multiple upstream chambers to the multiple downstream chambers under the conditions used in each one of the chambers. It will be appreciated that multiple separations of the same sample under the same conditions can also be carried out in this form of the apparatus according to the present invention.

Figure 10 is a schematic arrangement showing an embodiment based on Figure 8 but having multiple upstream chambers. In this configuration, it will be possible to carry out two-dimensional separation of macromolecules, using charge and molecular weight as variables. In this arrangement, it will be possible to determine the pI of a given protein by determining movement of the protein through the various separation membranes having decreasing molecular mass cut-offs at different pHs. In this situation, electrophoresis can be carried out in buffers having increasing pH from the left hand side through to the right hand side of the apparatus. After electrophoresis, the downstream chamber that contains the protein which has first moved through the membranes having molecular mass cut-offs greater than the protein would be indicative of the pI of the protein. It would also be possible to carry out multiple separations under different buffers to determine which proteins moved through to the downstream chambers under varying pH conditions.

A real advantage of the present invention is the ability to load very small samples and carry out fast separations without significant loss of the proteins or undue dilution of the samples. The ability to carry out dialysis of very small samples is also a distinct advantage for the same reasons.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Dated this eighteenth day of April 2000

Gradipore Limited
Patent Attorneys for the Applicant:

~~F B RICE & CO~~ ALLENS PATENT & TRADE MARKS

AUSTRALIAN PATENT OFFICE REG 22.10 (2)

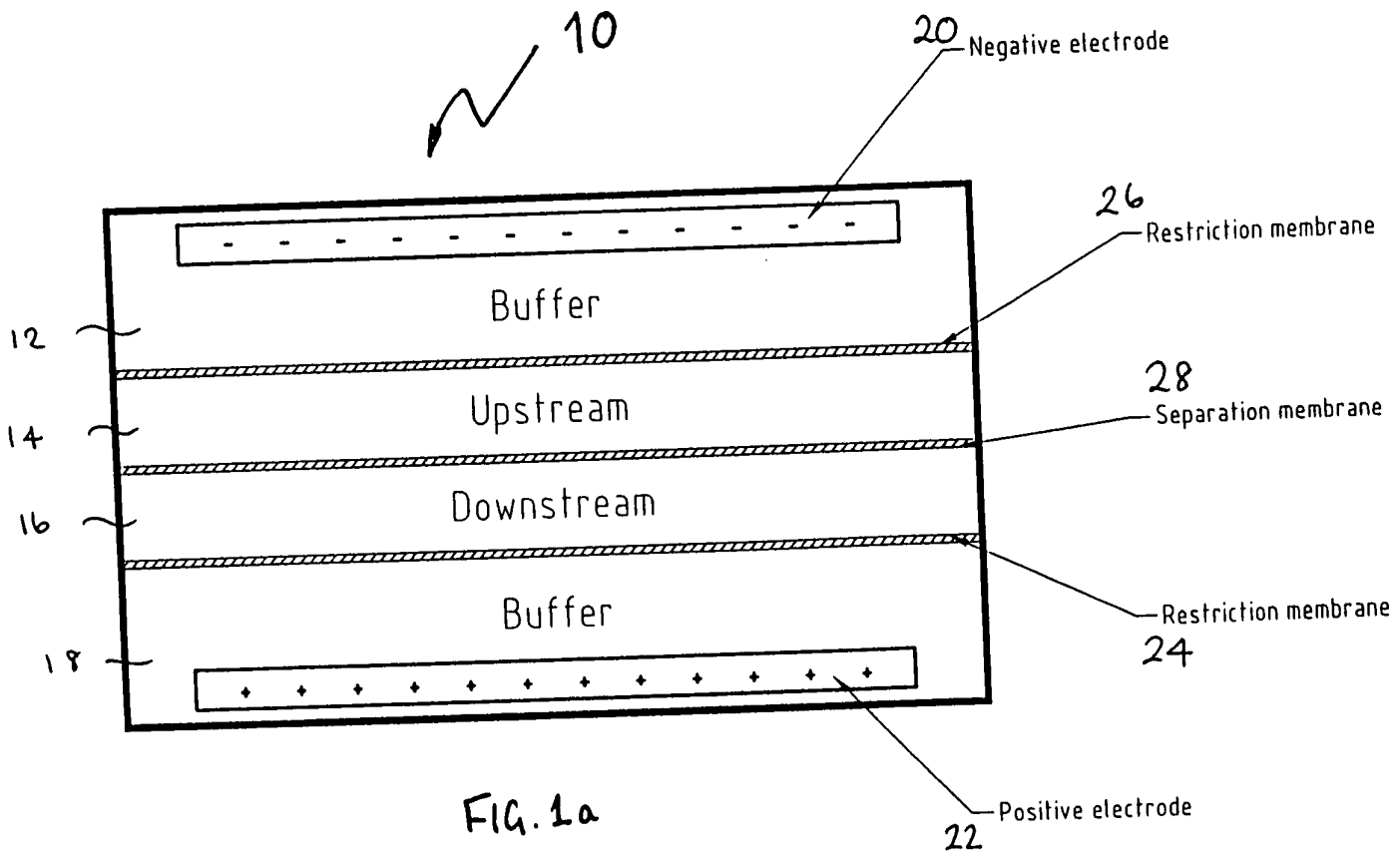

10
20
Negative electrode
26
Restriction membrane
Buffer
12
28
Separation membrane
Upstream
14
Downstream
16
Restriction membrane
24
Buffer
18
Positive electrode
22

FIG. 1a

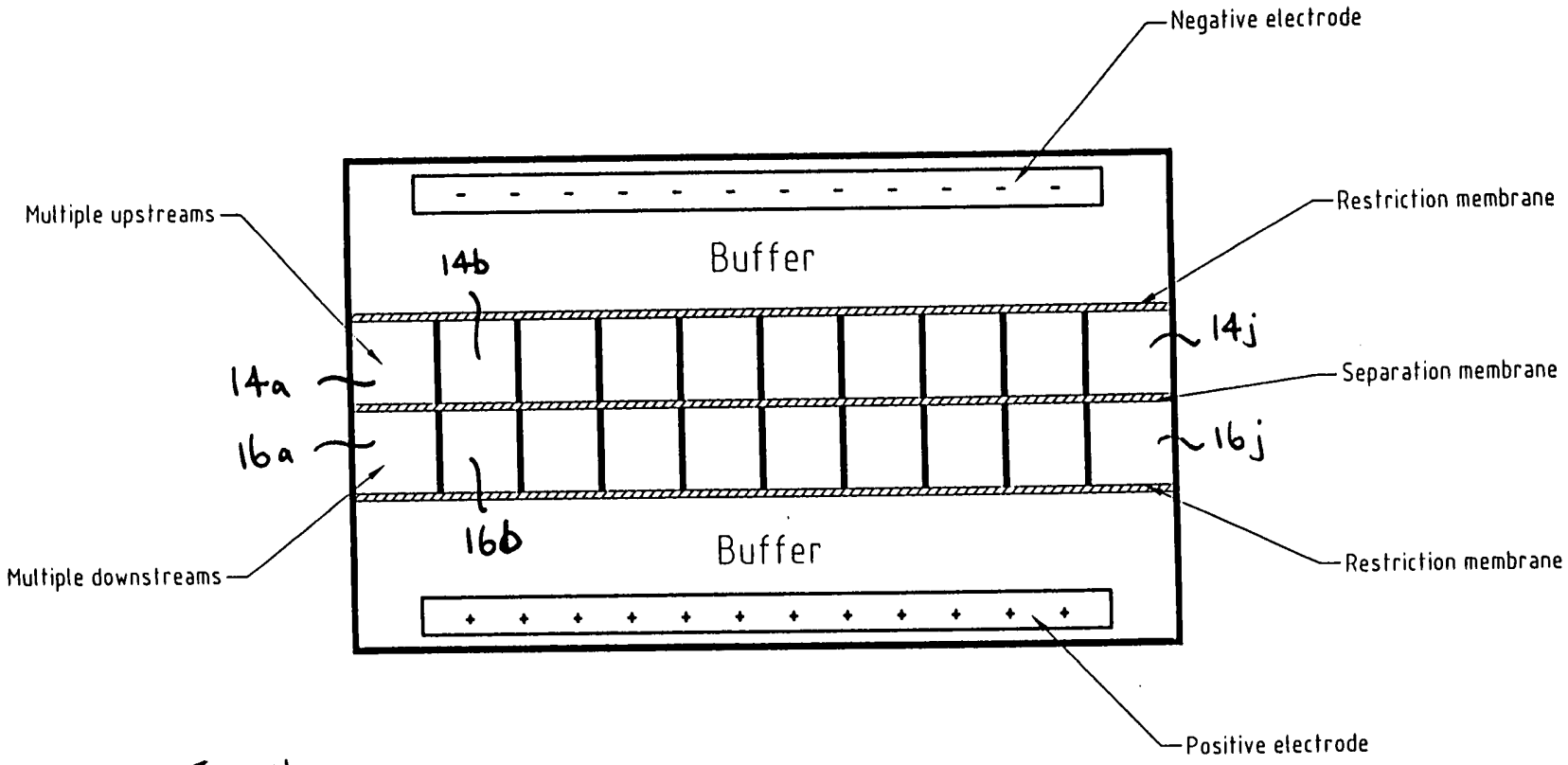

Negative electrode
Multiple upstreams
Restriction membrane
Buffer
14b
14j
14a
Separation membrane
16a
16j
16b
Buffer
Multiple downstreams
Restriction membrane
Positive electrode

FIG 1b

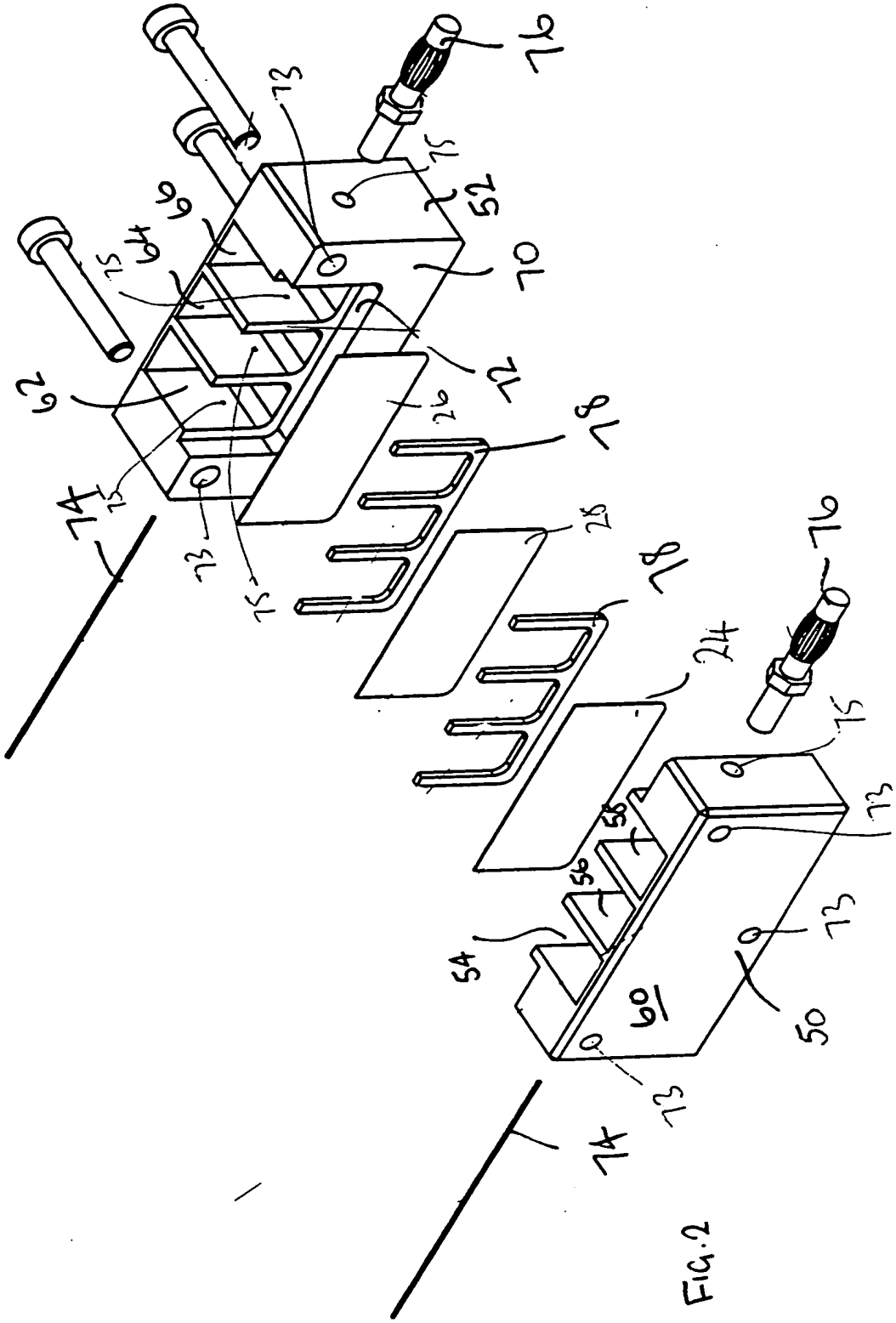


FIG. 2

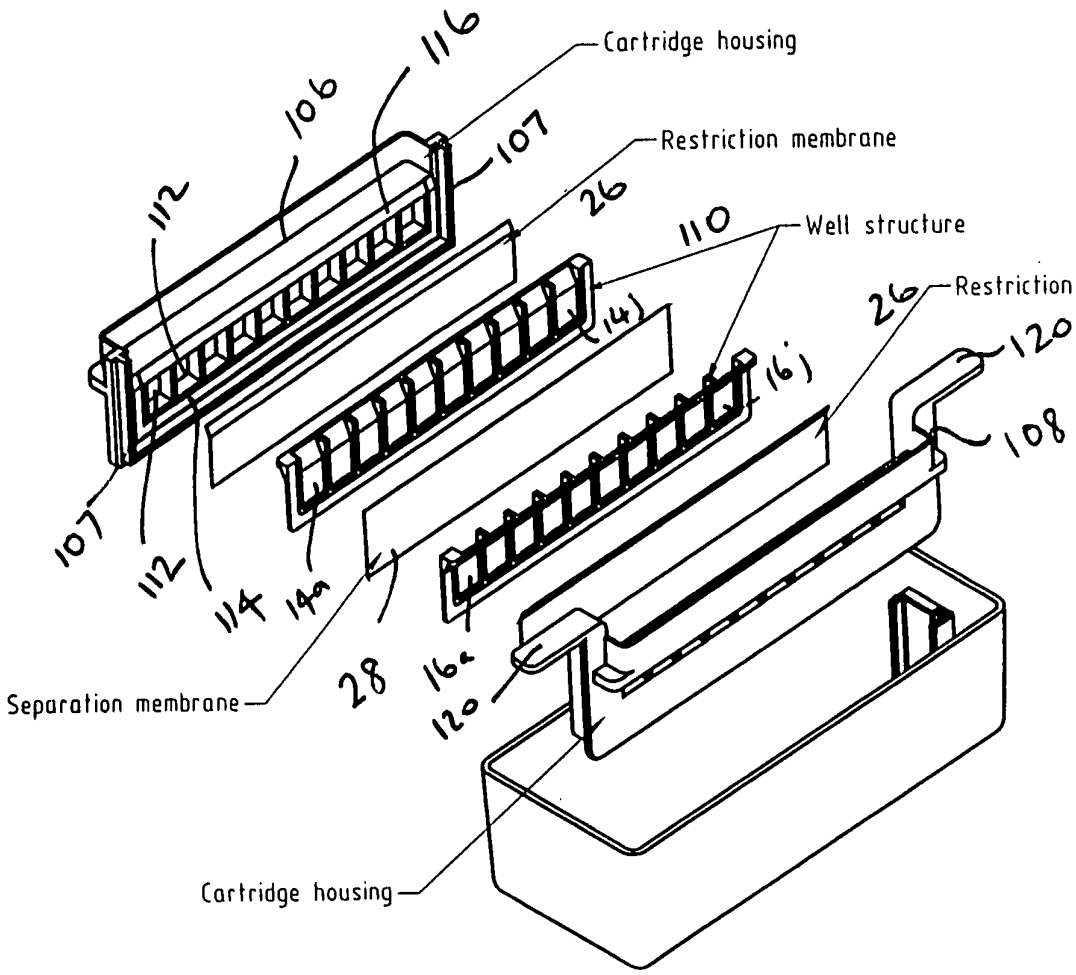
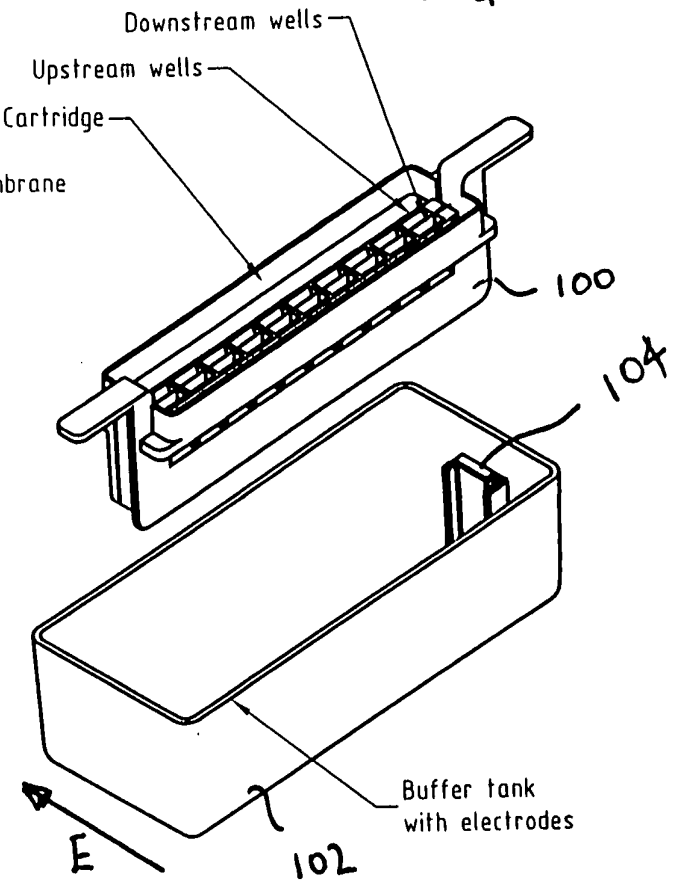

Cartridge housing
116
106
107
Restriction membrane
112
26
110
Well structure
(14)
(16)
26
Restriction membrane
120
108
107
112
114
14a
28
16a
120
Separation membrane
Cartridge housing

FIG. 3

FIG. 4


Downstream wells
Upstream wells
Cartridge
100
104
Buffer tank
with electrodes
E
102

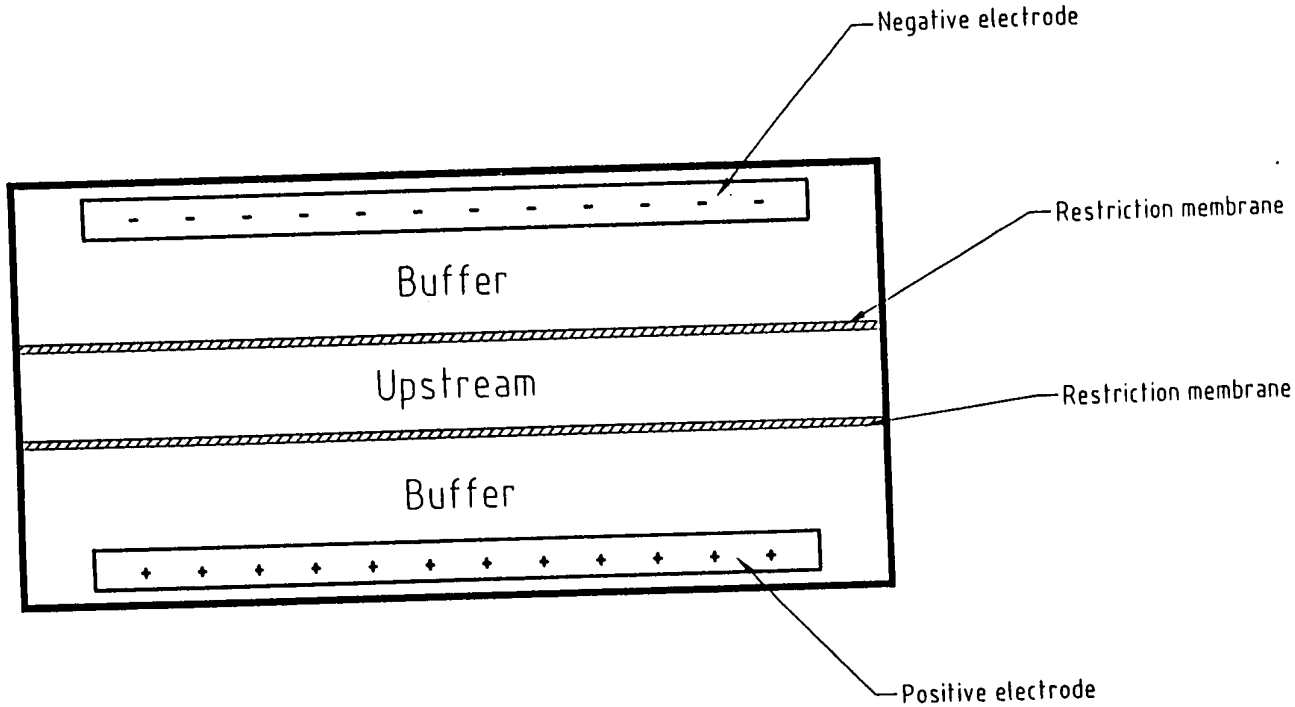

Negative electrode
Restriction membrane
Buffer
Upstream
Restriction membrane
Buffer
Positive electrode

Fig. 5

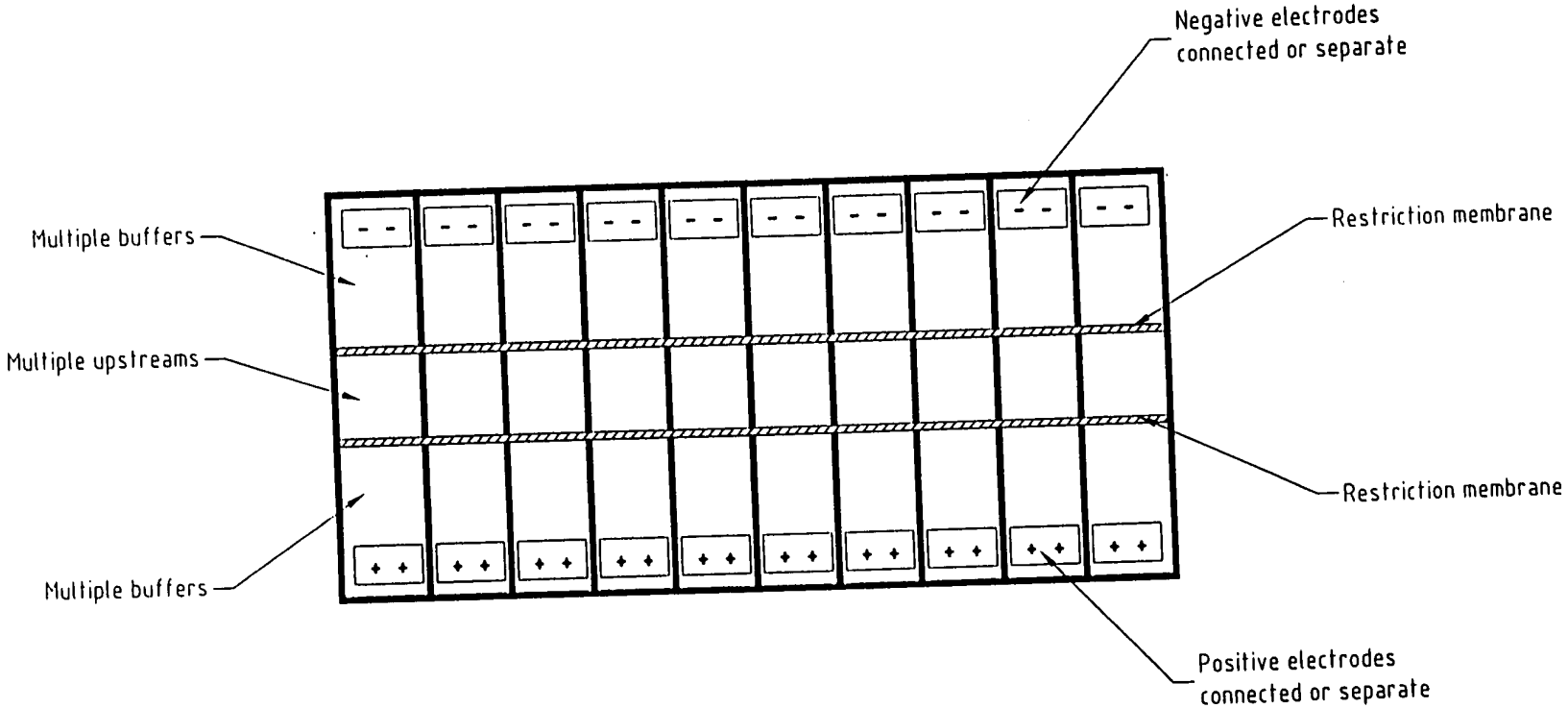

Negative electrodes
connected or separate
Multiple buffers
Restriction membrane
Multiple upstreams
Restriction membrane
Multiple buffers
Positive electrodes
connected or separate

FIG. 6

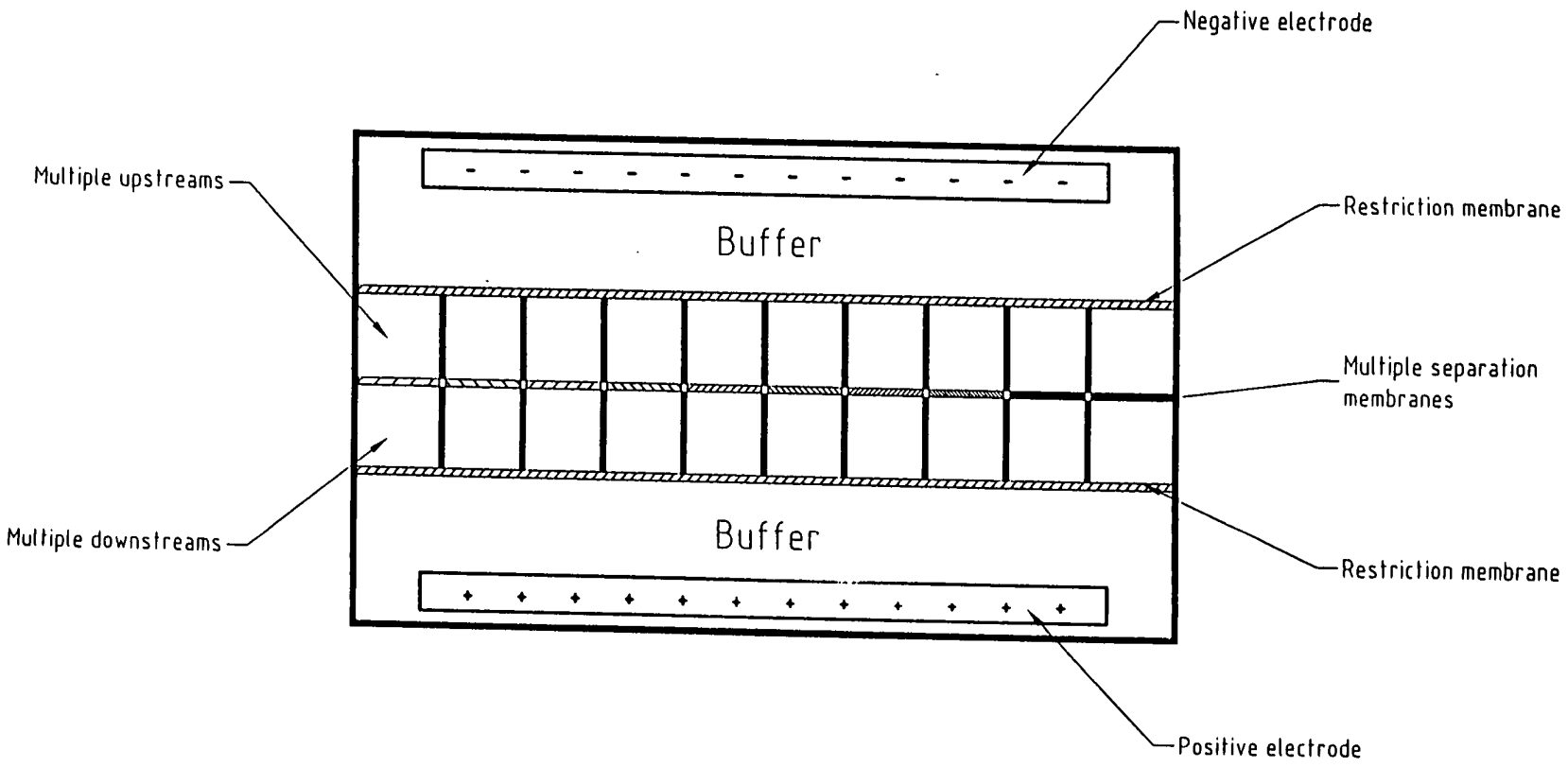

Negative electrode
Multiple upstreams
Buffer
Restriction membrane
Multiple separation membranes
Multiple downstreams
Buffer
Restriction membrane
Positive electrode

FIG 7

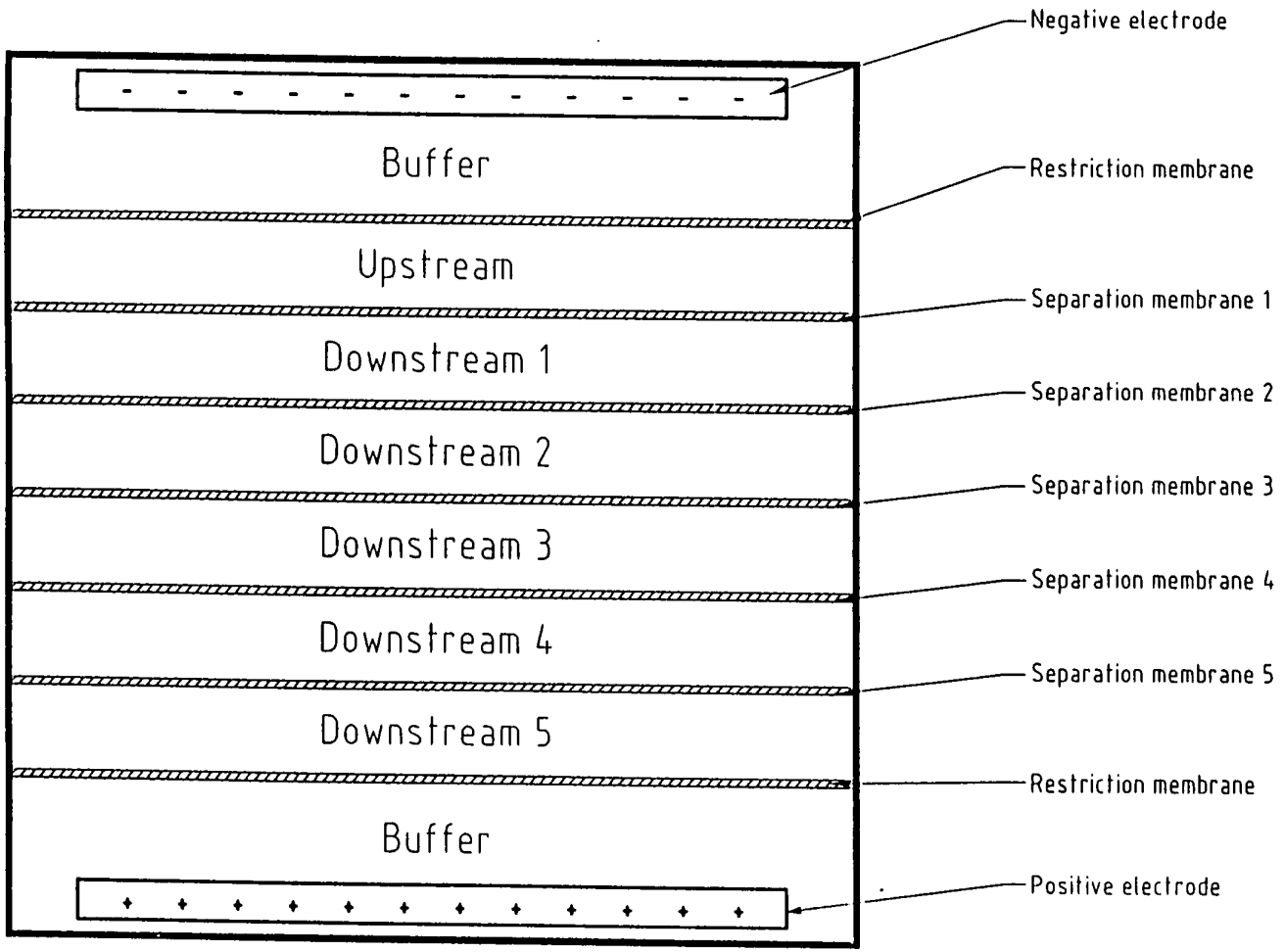

Negative electrode
Buffer
Restriction membrane
Upstream
Separation membrane 1
Downstream 1
Separation membrane 2
Downstream 2
Separation membrane 3
Downstream 3
Separation membrane 4
Downstream 4
Separation membrane 5
Downstream 5
Restriction membrane
Buffer
Positive electrode

FIG. 8

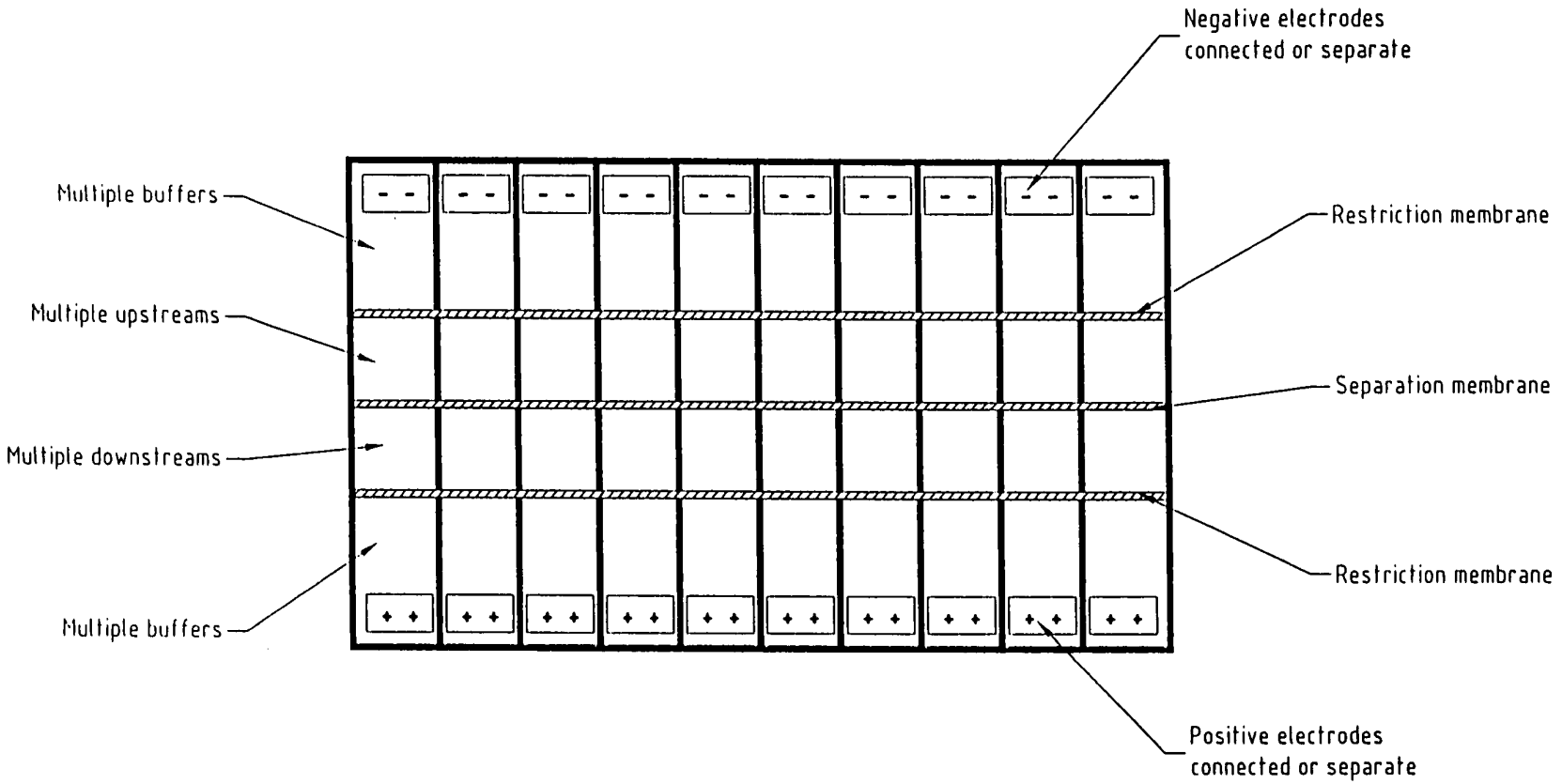

Negative electrodes
connected or separate
Multiple buffers
Restriction membrane
Multiple upstreams
Separation membrane
Multiple downstreams
Restriction membrane
Multiple buffers
Positive electrodes
connected or separate

FIG 9

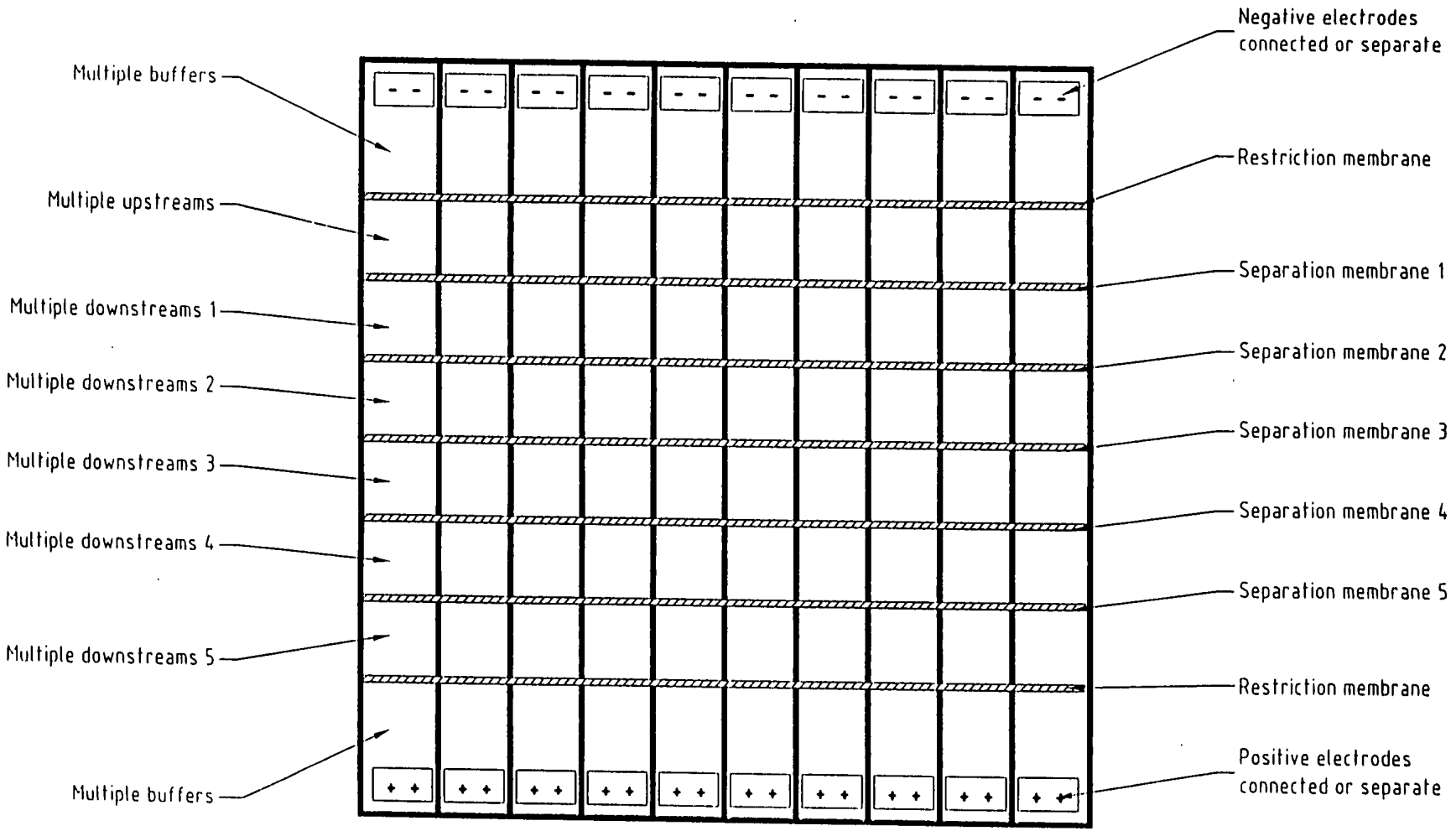

Multiple buffers
Multiple upstreams
Multiple downstreams 1
Multiple downstreams 2
Multiple downstreams 3
Multiple downstreams 4
Multiple downstreams 5
Multiple buffers
Negative electrodes connected or separate
Restriction membrane
Separation membrane 1
Separation membrane 2
Separation membrane 3
Separation membrane 4
Separation membrane 5
Restriction membrane
Positive electrodes connected or separate

FIG 10

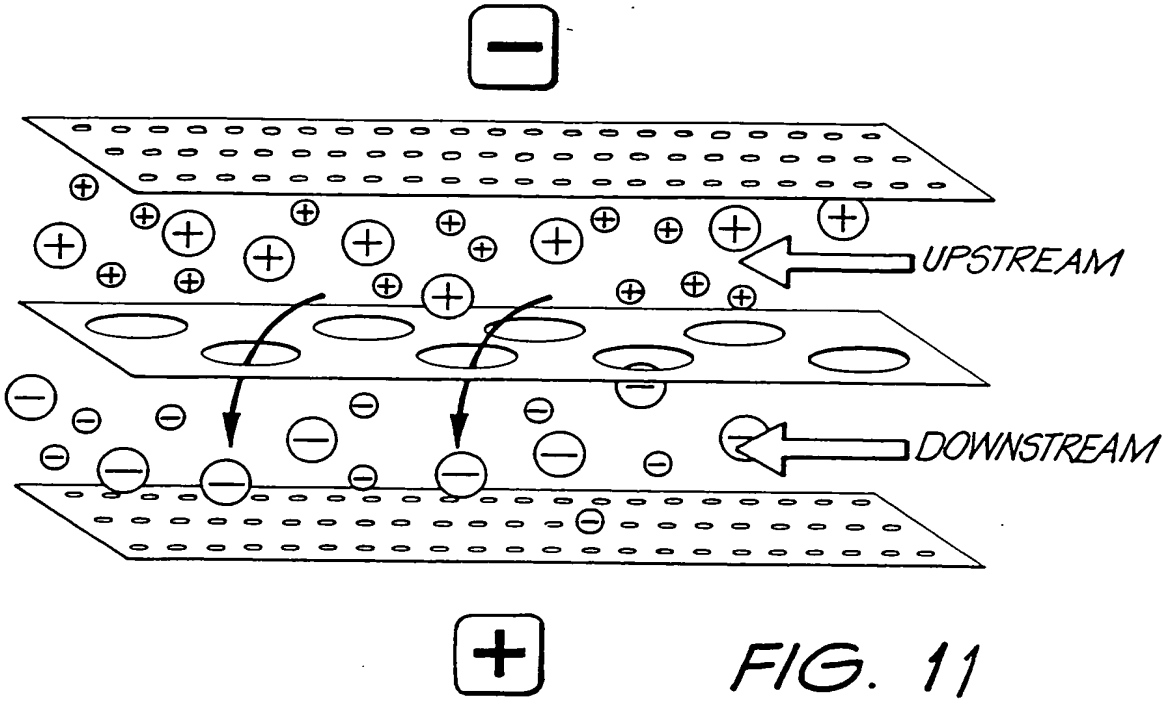


FIG. 11

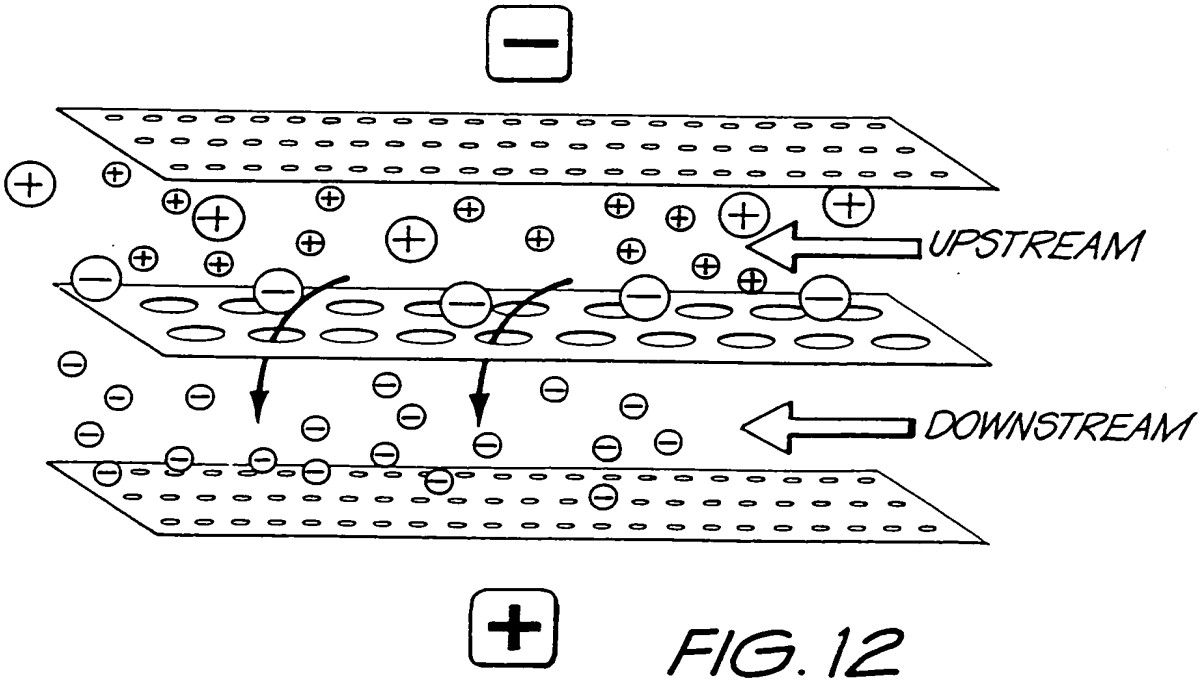


FIG. 12

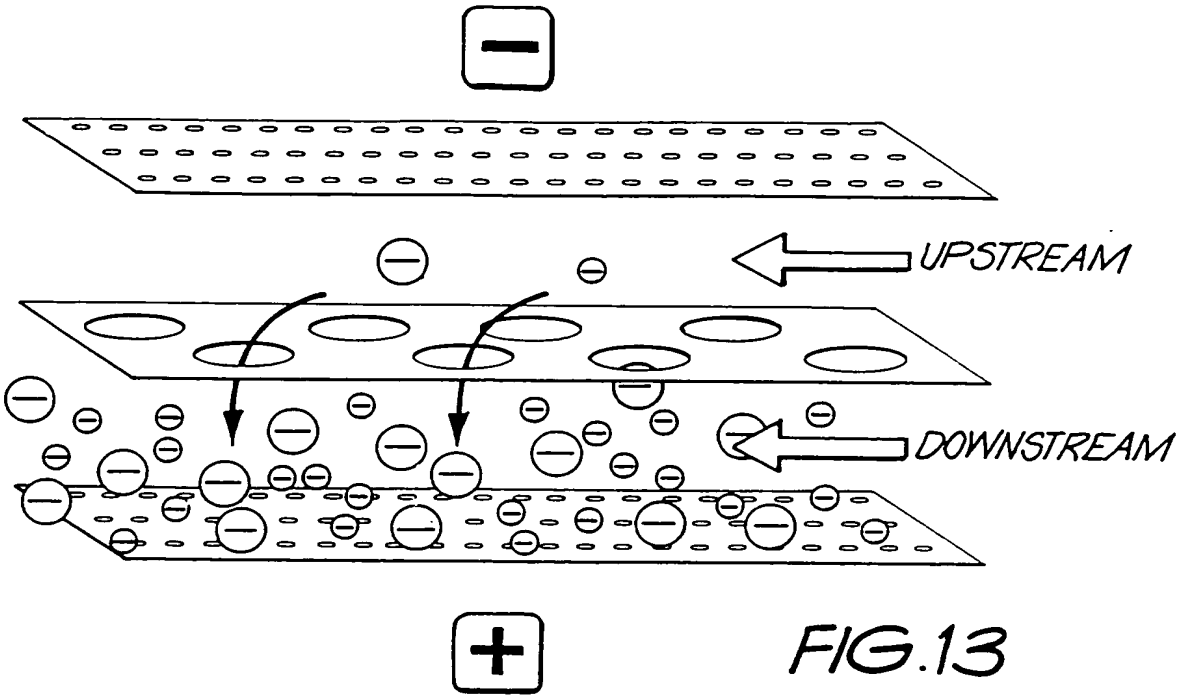


FIG.13

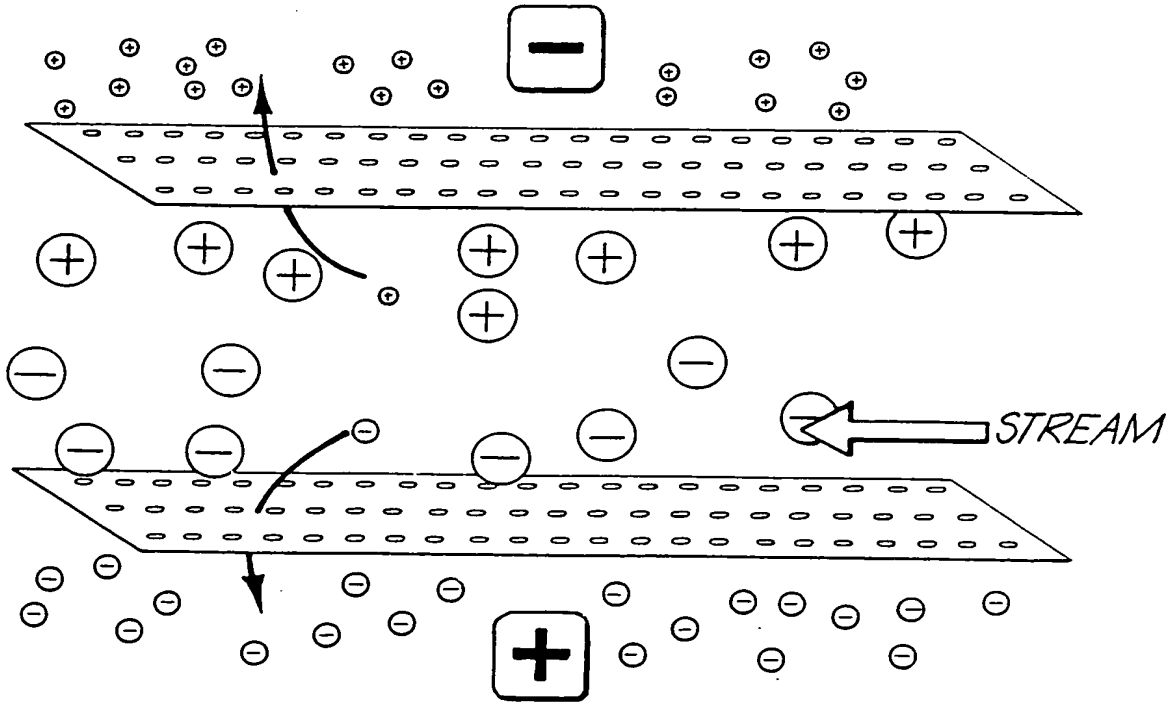


FIG.14